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From: Ungar, Susan
Sent: Tuesday, June 03, 2003 7:52 AM
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Subject: Papers for Examination of SN 09234290

Hi

I need the following papers to examine 09/234,290, this is a RUSH since this case is due this biweek.

1. Yoon et al, Annals of the NY Academy of Sciences, 2001, 928:200-211

2. Poulton et al, Diabetes/Metabolism Research and Reviews, 2001, 17(6)429-435 *****

3.Hanninen et al, Immunological Reviews, 2000, 173:109-119

4. Green et al (Immunological Reviews, 1999, 169:11-22

5. Simone et al, Diabetes Care, 1999,22 Suppl 2 B7-B15

6. Palmer, J. Clin. Investigation, 2001, 108(1)31-33

7. Seddon et al (Biochem Soc. Transactions,1997, 25(2)620-624)

8. Reddy et al, Histochemical Journal, 2000, 32(4)195-206

9. Ylinen et al, Pancrea, 2000, 20(2)197-205

10. Sainio et al, Pancrea, 1999, 18(3)282-293

11. Alamunits et al, Clinical and Experimental Immunology, 1999, 115(2)260-267.

I also need an entire volume, Cohen et al (Autoimmune Disease Models, A Guidebook, Academic Press, San Diego, 1994

Thanks
Susan Ungar
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703-305-2181
CM1-8B05

Arno Hänninen
Leonard C. Harrison

γδ T cells as mediators of mucosal tolerance: the autoimmune diabetes model

Authors' address
Arno Hänninen, Leonard C. Harrison,
The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Parkville, Australia.

Correspondence to:
Leonard C. Harrison
The Walter and Eliza Hall Institute of Medical Research
Post Office, The Royal Melbourne Hospital 3050
Victoria
Australia
Fax: 61 3 9347 0852
e-mail: harrison@wehi.edu.au

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Summary: Mucosal delivery of soluble antigen induces systemic tolerance and has been applied to the prevention of autoimmune diseases. We have studied mucosal tolerance in autoimmune diabetes using the non-obese diabetic mouse model. Treatment of prediabetic mice with the pancreatic islet autoantigen insulin, by aerosol or intranasal delivery, reduces the incidence of diabetes and is associated with induction of CD8 (αα) γδ T cells, small numbers of which prevent adoptive transfer of diabetes. We examine the evidence for γδ T cells in mucosal tolerance and discuss possible mechanisms underlying the induction and action of insulin-induced CD8 γδ regulatory T cells. CD8 γδ cells constitute the most abundant subpopulation of intraepithelial lymphocytes (IELs), the major lymphoid cell compartment and first line of cellular immune defence in the mucosa. Induction of regulatory CD8 γδ T cells requires conformationally intact but not biologically active insulin. In contrast, intranasal (pro)insulin peptide, or oral insulin which is degraded in the gut, induces CD4 regulatory cells. Regulatory γδ T cells secrete interleukin-10 in pancreatic lymph nodes, which could account for the antidiabetic and bystander suppressor effect of naso-respiratory insulin. The physiological role of γδ IELs in maintaining peripheral self-tolerance deserves further study.

Introduction

The immune system evolved as a defence against microorganisms potentially harmful to self. Self-tolerance, discrimination between self and non-self, is an essential property of the immune system. For T cells, it is first achieved during their development in the thymus by allowing only those precursors to mature that are not overly self-reactive. Nevertheless, many T cells with lower avidity for self escape this central selection mechanism and mature to be potentially autoreactive. Though these cells may be activated in different circumstances against self- or cross-reactive peptide epitopes, autoimmune disease is relatively uncommon. Several mechanisms exist for maintaining self-tolerance in the periphery. Apart from an inability of T cells to recognize cryptic antigen or be properly activated by antigen in the absence of non-specific co-stimulator molecules ('ignorance'), these mechanisms include functional silencing ('anergy') of T cells by modified presentation of antigen, antigen-

induced apoptosis of T cells ('deletion') and regulation of antigen-specific effector T cells by regulatory or suppressor cells.

While thymic tolerance is not readily amenable to manipulation in humans, mechanisms of peripheral tolerance are potentially inducible for the prevention and treatment of autoimmune disease. Mucosa-associated lymphoid tissues appear to be particularly adapted to maintaining systemic tolerance towards mucosally encountered antigens, using the same mechanisms responsible for peripheral tolerance in general (1, 2). Thus, delivery of antigens to the mucosa via oral, nasal or respiratory routes has in recent years attracted increasing attention as a means to downregulate systemic immunoinflammatory disorders. In this review, we examine the evidence that T cells expressing the γδ T-cell receptor (TCR) play an important role in mucosa-mediated tolerance. In particular, we focus on CD8 γδ T cells elicited by mucosal delivery of the autoantigen insulin, as regulators of T-cell-mediated pancreatic islet β-cell destruction in type 1 diabetes.

Localization, phenotype and cellular physiology of mucosal γδ T cells

Mucosal surfaces of the gastrointestinal, naso-respiratory and genitourinary tracts are constantly under threat from pathogens and potentially harmful substances, and the gut is frequently exposed to multiple food components. In addition to the physical barrier presented by mucosal epithelial cells, a local mucosal immune system plays a fundamental role in defence and self-tolerance. The mucosal immune system comprises several compartments: Peyer's patches, lymphocytes in the lamina propria, intraepithelial lymphocytes (IELs), and mesenteric lymph nodes that interface with the systemic immune system. Each of these has characteristics that serve a special purpose. Peyer's patches facilitate antigen uptake mainly via specialized epithelial cells called M cells and are rich in B cells for class switching into IgA-secreting cells (3). IgA secretion is one of the principal defences against invasive pathogens at mucosal surfaces. The lamina propria contains a high proportion of activated (interleukin (IL)-2R-α-positive) and memory (CD45RO-positive) antigen-experienced lymphocytes to allow rapid, preadapted immune responses against pathogens (4). IELs are scattered between mucosal epithelial cells close to the basement membrane. Although they do not form a discrete, organized lymphoid tissue, large numbers of IELs cover mucosal epithelia and they are the first lymphoid cells encountered by exogenous pathogens and antigens.

While Peyer's patches and the lamina propria resemble other lymphoid tissues in their T-cell subtype composition, IELs are distinct in mice and humans. Although phenotypically complex (5), IELs comprise predominantly CD8 and γδ T cells. Most intraepithelial γδ T cells are CD8 positive, but express the CD8 molecule as a homodimer of two α chains instead of the conventional αβ heterodimer (reviewed in (6–8)). In mice, γδ T cells appear earlier than αβ T cells in ontogeny in both thymus and intestine. They are produced from the foetal thymus in distinct waves, each of a particular TCR Vγ chain type. These monospecific γδ T cells populate different epithelia (reviewed in (9, 10)). The first wave expressing Vγ5 home to skin, where they are known as dendritic epithelial γδ T cells; the next expressing Vγ6 home to the female genitourinary tract and the tongue. After birth, γδ T cells are produced in the thymus in a more continuous fashion and comprise the majority of γδ T cells found in conventional lymphoid organs and blood. These cells preferentially express Vγ4 and Vγ1 (reviewed in (9)). Intestinal intraepithelial γδ T cells preferentially express Vγ7 and Vγ1 (reviewed in (9)) and develop at least partly in a thymus-independent manner (6–8). Intestinal γδ T cells as well as those in lymphoid organs differ from the early monospecific cells in that their TCR γ and δ chains have rearranged V, D and J regions. Although there are fewer V, D and J regions in γ and δ chains to rearrange than in α and β chains, N-nucleotides are inserted in up to three junctions of V, D and J regions (reviewed in (9, 10)) to create diversity equivalent to that of αβ T cells.

Recognition of heat shock proteins by isolated γδ T-cell clones (11, 12) and their localization has led to the idea that mucosal γδ T cells recognize stressed epithelial cells (13). In this view, they would serve a role intermediate between the innate and the adaptive immune systems. However, the diversity of their TCRs indicates an ability to recognize a multiplicity of distinct antigenic structures. Moreover, antigen recognition by γδ T cells is not necessarily governed by the rules for conventional αβ T cells, as γδ T cells can recognize even non-proteinaceous ligands (14) and either use unconventional (MHC class I like) restriction elements (reviewed in (10)) or recognize antigens directly without MHC (14).

Most IELs express the αEβ7 intregrin (CD103), which binds to epithelial cell E-cadherin (15, 16) and is required for target cell lysis by IELs, many of which are constitutively cytotoxic *ex vivo* (reviewed in (9)). Cytotoxic IELs would provide the first line of cellular defence against pathogens. It is also likely that γδ IELs have a role in maintaining epithelial homeostasis. Both skin and intestine-derived γδ T cells are able to support epithelial cell growth by secreting keratinocyte growth factor (17). The turnover of epithelial cells and their unique constitutive MHC class II expression are reduced in TCR δ gene knockout mice (18). γδ IELs can also make a multiplicity of cytokines,

including IL-2, interferon (IFN)-γ, tumour necrosis factor-α, IL-4, IL-10 and transforming growth factor (TGF)-β (reviewed in (9)). Therefore, they may regulate the growth and differentiation of intestinal epithelial cells (19) in concert with autocrine and paracrine factors secreted by epithelial cells themselves and factors derived from the underlying mesenchyme (20). γδ T cells are also present in nasal-associated lymphoid tissue and the bronchial-associated lymphoid tissue of the respiratory tract (21), where they are even less characterized due partly to the lower number of accessible cells in these sites (22).

Mechanisms of mucosal immunoregulation

The notion that delivery of soluble protein antigens to mucosal surfaces induces tolerance is based on historic observations that exposure via the oral route induces antigen-specific hyporesponsiveness upon systemic rechallenge. The first formal studies of oral tolerance, carried out by Wells in guinea pigs early this century (23), showed that feeding ovalbumin (OVA) prevented systemic anaphylaxis to OVA. Insight into the immune basis of this phenomenon came later with the demonstration by Chase (24) that feeding the hapten 2,4-dinitrofluorobenzene (DNFB) suppressed contact sensitivity responses to DNFB. Although the outcome of oral tolerance is generally considered to be suppression of cell-mediated immunity, as reflected in classic contact and delayed-type hypersensitivity (DTH) responses, seminal studies (reviewed in (25)) showed not only that DTH but also IgE responses were suppressed after oral antigen, an observation seemingly at odds with the popular Th1-Th2 paradigm of immune responses. More recently, mechanisms responsible for mucosa-mediated tolerance have been identified. In mice bearing transgenic CD4 T cells specific for OVA, large doses of oral OVA induced apoptosis and deletion of OVA-specific T cells, whereas small doses induced local expansion of OVA-specific T cells in Peyer's patches and was associated with production of the Th2/'anti-inflammatory' cytokines, IL-4, IL-10 and TGF-β (26). Others found that feeding large doses of OVA simultaneously induced apoptosis, decreased proliferative and cytokine responses, and induced secretion of TGF-β in OVA-specific transgenic CD4 T cells (27). Oral feeding of repeated small but not large doses of myelin basic protein (MBP) to mice bearing transgenic CD4 T cells specific for an MBP peptide induced secretion of IL-4, IL-10 and TGF-β by these cells (28).

Studies in animal models of experimentally induced autoimmune disease, including experimental allergic encephalomyelitis (EAE), collagen- and adjuvant-induced arthritis and autoimmune uveitis, indicate that exposure of mucosa to antigen may be an effective means to prevent and even suppress ongoing autoimmune disease (reviewed in (29)). These models are induced with a defined antigen which when administered mucosally (or by other 'tolerizing' means) suppresses disease by mechanisms that target antigen-specific, pathogenic-effector T cells. However, human autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, type 1 (insulin-dependent) diabetes and others occur in outbred populations and are not associated with a single, defined autoantigen, suggesting it may be more difficult to target pathogenic T cells by mechanisms such as anergy or deletion. In spite of this, mucosal administration of a single autoantigen, (pro)insulin, to the non-obese diabetic (NOD) mouse, a model of spontaneous autoimmune pancreatic islet β-cell destruction, delays the onset and reduces the incidence of diabetes. The NOD mouse shares a number of key features with humans who develop type 1 diabetes, including at least two autoantigens and a structurally similar class II MHC molecule (*Table 1*). Administration of oral insulin (30, 31), aerosol insulin (32), intranasal insulin (33), (pro)insulin peptides (33, 34) or glutamic acid decarboxylase (GAD) peptides (35), all at least partially inhibit β-cell destruction in NOD mice. Thus, mucosal delivery of a single autoantigen in NOD mice may induce tolerance against pathogenic T cells of more than one antigen specificity. This phenomenon, associated with induction of regulatory cells that can transfer suppression to non-treated animals, is referred to as active tolerance with 'bystander' suppression (1, 2, 29).

The role of γδ cells in mucosal immunoregulation

The phenotype of the regulatory cells induced by mucosal antigen that can transfer tolerance and downregulate pathogenic autoimmunity varies in different models and studies. Tolerance induced in NOD mice by oral insulin (30, 31) or GAD peptides (35) was transferable by CD4 T cells. On the other hand, oral MBP or proteolipid that prevent EAE in SJL mice induced both CD4 and CD8 regulatory cells (36), and oral MBP that prevents EAE in Lewis rats induced CD8 regulatory cells (37, 38). The TCR class of these CD8 regulatory cells was not defined.

Several lines of evidence indicate that γδ IELs regulate autoimmunity (*Table 2*). γδ T cells have been implicated in the downregulation of immune responses in various inflammatory disorders (46–51) and may acquire immunoregulatory properties at the mucosal level. Holt and co-workers (52) immunized different strains of rats and mice systemically to OVA to induce specific IgE antibodies and airway hyper-responsiveness to inhaled OVA. They found that administration of OVA to the res-

Table 1. The NOD mouse as a model of human type 1 diabetes

Feature	NOD mouse	Human
Onset in youth	yes	yes
Gender bias	diabetes F > M insulitis F = M	F = M
Genetic susceptibility		
MHC class II β57 non-Asp	I-Aᵍ⁷	DQ8(*0302)
Polygenic non-MHC	yes	yes
Environmental influence on gene penetrance	yes	yes
Disease transmission via bone marrow	yes	yes
Mononuclear cell infiltration of		
Islets (insulitis)	marked	moderate
Other organs	yes	sometimes
Impaired immunoregulation	yes	yes
Expression of endogenous retrovirus in islets	yes	?
Autoantigens: (pro)insulin, glutamic acid decarboxylase (GAD)	yes	yes
Response to autoantigen-specific therapy	yes	?

piratory mucosa in the form of an aerosol or by intranasal application before systemic immunization suppressed subsequent OVA-specific IgE antibody and IL-4 responses by an IFN-γ-dependent mechanism (53). The effects of aerosol or intranasal antigen were transferable to untreated mice by small numbers of CD8 γδ T cells (54), indicating that these cells most likely mediated tolerance in this model. Oral OVA has also been associated with the induction of regulatory CD8 γδ T cells. Initially, Ke & Kapp (55) showed that the inhibitory effect of oral OVA on priming of CD4-dependent antibody responses and cytotoxic CD8 T-cell responses was mediated by CD8 T cells. In a subsequent study (44) tolerance induction was abolished by pretreating animals with an antibody (GL3) that blocks the function of γδ T cells. Furthermore, regulatory cells were not induced in mice genetically deficient in γδ T cells, implicating CD8 γδ T cells as the regulatory cell. Mengel et al. (45) had also found that oral tolerance to OVA was abrogated by blocking γδ T-cell function with GL3 antibody. In NOD mice, we found that intraperitoneal GL3 prevented induction by aerosol insulin of regulatory CD8 T cells that block adoptive transfer of diabetes by effector T cells (33). In contrast to these consistent observations, Seymour et al. (56) reported that suppression of OVA-specific IgE responses occurred after aerosol OVA in mice pretreated with an antibody (2.43) that blocks the function of CD8 T cells, as well as in mice deficient in γδ T cells. However, it was not determined whether these mice lacking functional CD8 or γδ T cells were able to generate regulatory cells capable of transferring tolerance. γδ T cells with a contrasuppressive effect have also been described by one group: oral tolerization of mice with sheep red blood cells was abrogated by transfer of γδ T cells derived from the gut intraepithelial compartment after immunization of mice with the same antigen (57). γδ T cells, like αβ T cells, presumably can have multiple functions depending on conditions and circumstances.

In summary, several independent studies have implicated γδ T cells as mediators of mucosa-mediated tolerance. However, other T cells are also known to have a similar regulatory role. Redundancy would not be unexpected for a function as crucial and complex as peripheral immune regulation. At present, the determinants of regulatory cells found in different models are not clear, but we have addressed this issue in regard to the form and route of delivery of insulin in the NOD mouse and propose an explanation below.

γδ T cells and suppression of autoimmune diabetes after intranasal or aerosol insulin

(Pro)insulin appears to be a key autoantigen that drives T-cell-mediated destruction of pancreatic islet β cells in type 1 diabetes (*Table* 3). Administration to NOD mice of oral insulin (30, 31) or intranasal insulin B-chain peptide aa 9-23 (34, 58) or proinsulin B-C chain peptide aa 24-36 (33) decreases diabetes incidence. Oral insulin was associated with the induction of CD4 T cells that adoptively transfer tolerance (31). Intranasal insulin B chain aa 9-23 suppressed primed lymph node responses to itself, which was postulated but not shown to be due to the induction of regulatory cells (34). However, intranasal proinsulin B-C chain aa 24-36, which binds to the NOD mouse MHC class II molecule I-A^{g7}, induced CD4 regulatory T cells (33). Intranasal GAD65 T-cell epitope peptides sup-

Table 2. Evidence that γδ IELs regulate autoimmunity

Germ-free NOD mice have an increased incidence of diabetes that is decreased by conventional conditions of housing and feeding (39).
Bacterial colonization dramatically increases thymus-derived and thymus-independent IELs in germ-free strains (44, 41).
Neonatal (3 day) thymectomy in mice leads to organ-specific autoimmune disease (42) and is associated with failure to develop IELs (43).
γδ T cells are necessary for the induction of oral (44, 45) and naso-respiratory tolerance (33).
Aerosol insulin induces CD8 γδ T cells that prevent adoptive transfer of diabetes in NOD mice (32).

Table 3. Evidence for (pro)insulin as a key autoantigen in type 1 diabetes

(Pro)insulin is the only β-cell-specific autoantigen in type 1 diabetes.
Insulin-specific clones represent the majority of T-cell clones isolated from pancreatic islets of NOD mice (58).
Administration of insulin (30, 33, 59) or (pro)insulin peptides (33, 34) by various routes (mainly mucosal) suppresses diabetes incidence in NOD mice.
NOD mice expressing proinsulin II as a transgene in antigen-presenting cells under the control of an MHC class II promoter do not develop β-cell autoimmunity (60).
Polymorphism of human insulin gene: *IDDM2* maps to 5′ VNTR (11p15) and regulates proinsulin expression in pancreas and thymus (61, 62).
Autoantibodies to insulin predict time to diabetes and correlate inversely with age at diagnosis (63).
T cells to insulin (64) and proinsulin B24-C36 (65) identify at-risk humans.

pressed splenic T-cell responses to GAD and other autoantigens, and was associated with immune deviation from IFN-γ to IL-5 secretion and with induction of CD4 T cells that adoptively transferred tolerance (35).

We delivered intact insulin as an inhaled aerosol or intranasally, or peptides from proinsulin intranasally, to delay the onset/reduce the incidence of diabetes in female NOD mice (32, 33). Aerosol treatment for 10 days and then weekly for 10 weeks, from 4 weeks of age, reduced diabetes incidence at 250 days of age from 80–90% to 30–40% (32). The same outcome is achieved even when treatment is started at 8 weeks of age when the mouse's immune system is sensitized to both proinsulin and GAD, consistent with the induction of regulatory cells capable of active bystander suppression. Indeed, the protective effect of aerosol insulin was transferable into non-treated recipients with spleen cells from treated mice co-transferred with 'diabetogenic' effector cells, and was associated with reduced proliferative responses of T cells to both the insulin B-chain epitope (aa 9-23) and GAD65 (32). Apart from bystander suppression, the reduced T-cell response to GAD may reflect less β-cell destruction and therefore less T-cell sensitization to GAD in insulin-treated mice. Consistent with immune deviation, splenocytes from protected mice secreted more IL-4 and IL-10 in response to the insulin B-chain epitope than cells from control, OVA aerosol-treated mice. To determine the phenotype of the regulatory cells identified by adoptive co-transfer, selective depletion of either CD4 or CD8 T cells and positive sequential selection of CD8 and γδ T cells was performed (32). This revealed that small numbers of purified CD8 γδ T cells ($1–2 \times 10^5$) completely accounted for the ability of splenocytes from insulin aerosol-treated mice to block adoptive transfer of diabetes (*Fig. 1*). These CD8 γδ T cells express CD8 αα and are phenotypically IELs. Not surprisingly, therefore, depletion of conventional CD8 αβ cells by anti-CD8β antibody plus complement did not abrogate the regulatory effect of splenocytes from insulin aerosol-treated mice.

Insulin was administered intranasally by direct application (10 μl, 4 mg/ml) to the nose of unanaesthetized female NOD mice. Compared to the aerosol route, intranasal is a more strictly defined route of antigen delivery. Intranasal antigen is reported to drain selectively to the superficial (anterior) cervical lymph nodes (reviewed in (66)). Consistent with this, we found that proliferation of transgenic CD4 (OT-II) and CD8 (OT-I) T cells specific for OVA epitopes is restricted to the anterior cervical lymph nodes in mice given intranasal OVA (A. Hänninen, L. C. Harrison, unpublished). As with aerosol insulin, intranasal intact insulin also reduced diabetes incidence in NOD mice, and was associated with the induction of CD8 T cells that suppress adoptive transfer of diabetes (33). Thus, both aerosol and intranasal insulin induce regulatory CD8 γδ T cells, whereas oral insulin induces CD4 regulatory T cells (31, 67). By using OVA-specific, fluorescent (carboxyfluorescein diacetate succinimidyl diester (CFSE) dye)-labelled transgenic CD4 (OT-II) T cells in an adoptive transfer system, we found proliferation in mediastinal and anterior cervical lymph nodes but not in gut-associated (mesenteric) lymph nodes, after aerosol OVA (A. Hänninen, L. C. Harrison, unpublished). This indicates that aerosolized antigen drains to lymph nodes of the respiratory, not gastrointestinal, tract.

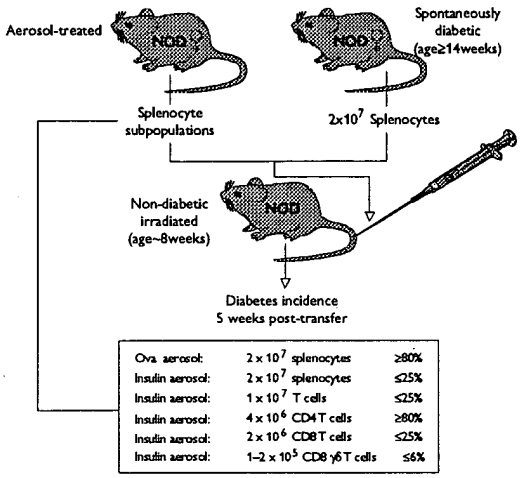


Fig. 1. Adoptive transfer of diabetes is suppressed by CD8 γδ T cells induced by aerosol insulin in NOD mice. Recombinant human insulin or control OVA protein at 4 mg/ml were delivered over 10 min at an air flow rate of 6 l/min in a rated droplet size of <5.8 μm to groups of 24–32 mice. NOD female mice were treated for 3–10 consecutive days from 28 days of age, then weekly. At 105 days of age, when the incidence of diabetes was 6.3% in the insulin-treated group and 25% in the OVA-treated group, splenocytes from at least six mice per group were pooled, fractionated by MACS MicroBeads (Miltenyi, Biote, Bergisch-Gladbach, Germany) and co-transferred intravenously with splenocytes from older, spontaneously diabetic NOD female mice into young, irradiated NOD male mice. The onset of diabetes was then monitored by measuring blood glucose weekly starting 2 weeks after transfer.

Mechanisms of γδ T-cell-mediated suppression of diabetes

How CD8 γδ T cells induced by aerosol/intranasal insulin suppress diabetes is unknown, but γδ T cells possess several properties that may explain this observation. They secrete a range of both Th1 and Th2-type cytokines and are constitutively cytotoxic (reviewed in (9)). Anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β implicated in the regulatory effects of some CD4 T cells in oral tolerance (26–28) (reviewed in (1)) may also mediate immunoregulation by CD8 γδ T cells. Aerosol OVA-induced tolerance against airway hyper-responsiveness to OVA was associated with increased IFN-γ production by CD8 γδ T cells (54). IFN-γ might suppress an immune response characterized by IL-4-mediated secretion of IgE and eosinophilia, but it is not clear how it would suppress β-cell destruction in type 1 diabetes, a Th1-cell (i.e. IFN-γ)-dependent process (68, 69). As mentioned, splenocytes from insulin aerosol-treated NOD mice secreted significantly more IL-4 and IL-10 than control cells (32). Subsequently, by intracellular labelling, we have detected IL-10-containing γδ T cells in pancreatic lymph nodes, but not islets, of aerosol insulin-treated mice (*Fig.* 2).

IL-10 is a potent immunoregulatory cytokine, and cloned T cells, termed T regulatory type 1 (Tr1), that secrete large amounts of IL-10 have been shown to suppress autoimmune colitis in the severe combined immunodeficiency disease (SCID) mouse (70). Islet-reactive T cells transduced with IL-10 can suppress cyclophosphamide-accelerated β-cell destruction in NOD mice (71). The elaboration of IL-10 by small numbers of activated γδ T cells in pancreatic lymph nodes, where pathogenic T cells are probably activated, represents a mechanism for suppressing destructive autoimmunity by immune deviation and/or downregulation of antigen presentation or co-stimulatory activity of antigen-presentating cells (APCs). In addition, we speculate that γδ T cells could also operate by other mechanisms related to their cytotoxic potential and/or recognition of unusual restriction elements or activation markers, as illustrated schematically in *Fig.* 3.

How does insulin induce regulatory γδ T cells?

We have begun to examine how γδ T cells are triggered by insulin. We wished to show first that insulin is recognized as an antigen and does not stimulate γδ T cells as a hormone/growth factor via insulin receptor-mediated signalling. The following questions were then posed. If indeed it is an antigen, is insulin recognized directly as the native protein or does recognition require processing by an APC? Is the APC a 'professional' dendritic cell in the mucosa/mucosal lymph node, or is it the mucosal epithelial cell which expresses both MHC class I and II as well as the non-polymorphic class 1-like CD1 and thymus leukaemia (TL) antigen molecules? Which restriction molecule is used for presentation? Alternatively, are γδ T cells triggered indirectly, e.g. after activation by insulin of conventional CD4 αβ T cells which could then stimulate γδ T cells via activation markers like heat-shock proteins or the TL antigen (72)?

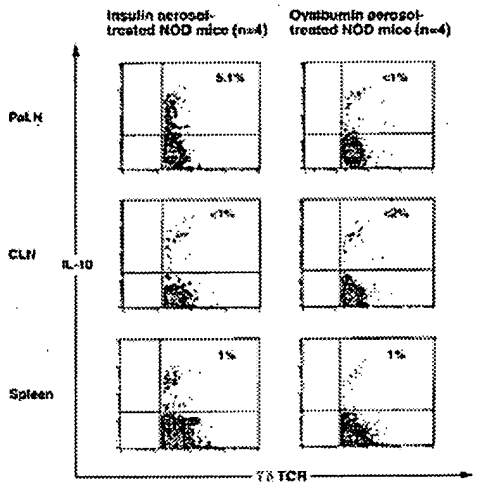


Fig. 2. γδ T cells isolated from pancreatic lymph nodes of insulin aerosol-treated mice express IL-10. NOD female mice aged 56 days were treated on 3 alternate days with either aerosol insulin (left) or ovalbumin (right, control treatment). Fourteen days after the first treatment, lymphocytes were isolated from pancreatic lymph nodes (PaLN) and cervical lymph nodes (CLN), incubated for 4 h with phorbol myristate acetate and ionomycin and, during the last 2 h, with brefeldin A. After washing, they were surface-labelled with fluorescein isothiocyanate-conjugated GL3 (anti-γδ) hamster monoclonal antibody, fixed and permeabilized, and then intracellularly labelled for either IL-10 (shown), or IL-4, IL-2 or IFN-γ (not shown) with phycoerythrin-conjugated antibodies. In the upper right corner of each box are net % positive stained cells obtained after subtracting the isotype-matched control.

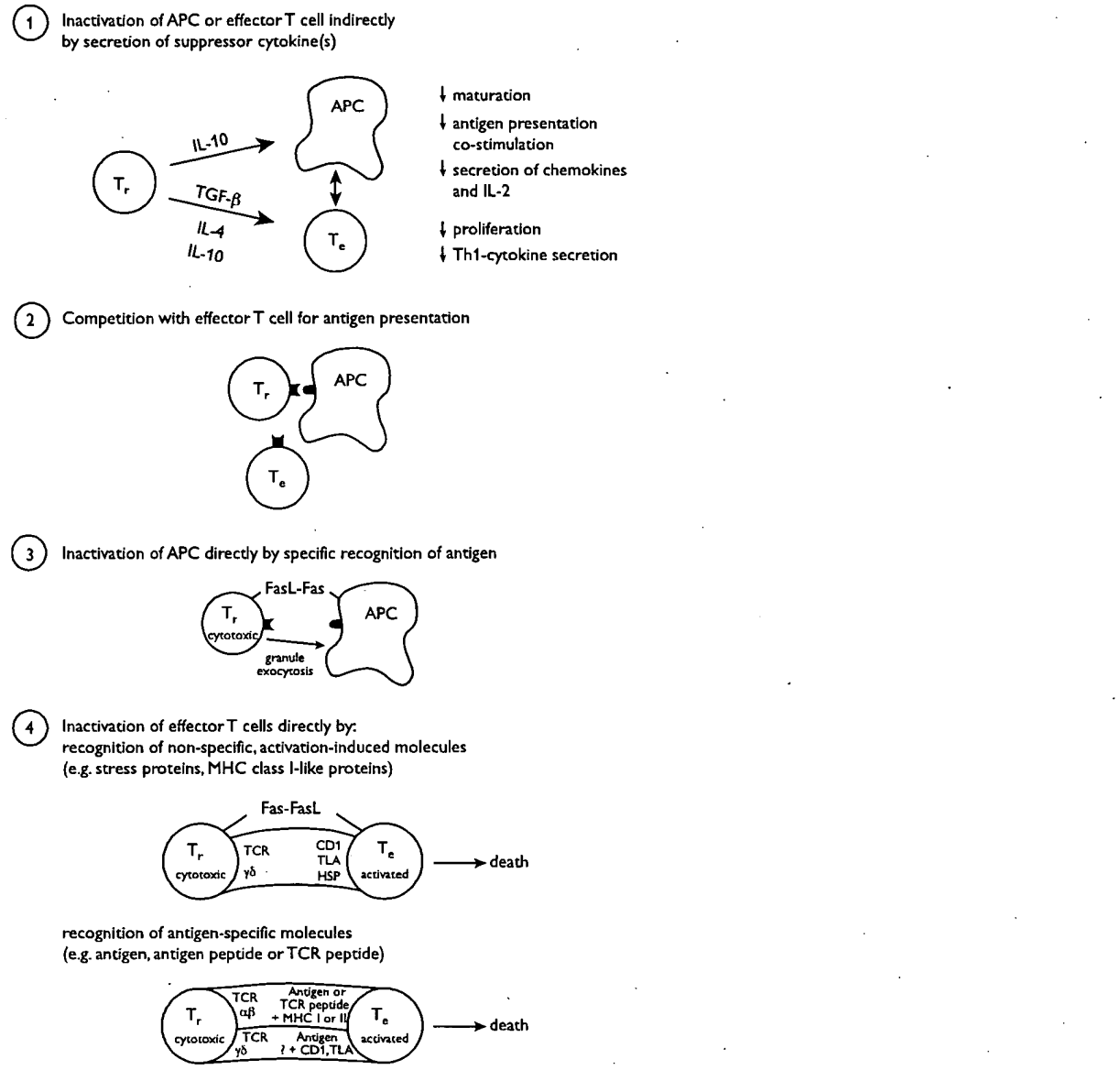
Insulin poses a particular problem as a tool for immunoregulation because it may stimulate lymphocytes not only through their antigen receptors but also through its own receptor. Activated lymphocytes upregulate insulin receptors and may therefore be particularly sensitive to insulin. A substrate of the insulin receptor kinase-1 (IRS-1) is closely related to the substrate 4PS of a kinase activated by IL-4 receptor signalling, and insulin causes tyrosine phosphorylation of 4PS in haematopoietic cells (73, 74). If insulin mimicked IL-4 in downstream signalling it might promote T-cell commitment to Th2 differentiation, although direct evidence for this is lacking. To test if there was a requirement for bioactivity of insulin in inducing regulatory cells, we used an inactive form of insulin, B25 Asp insulin (provided by Dr Thomas Dyrberg, Novo Nordisk, Copenhagen), in which a substitution of phenylalanine for aspartic acid at position 25 of the B chain abolishes receptor binding. Given intranasally to female NOD mice, B25 Asp insulin was as effective as native insulin in inducing regulatory CD8 T cells (33). We conclude then that insulin behaves as an antigen, not as a hormone, to induce regulatory CD8 γδ T cells.

Trafficking of CD8 γδ T cells to other sites (draining lymph nodes, pancreatic lymph nodes/islets, spleen) after induction in the naso-respiratory mucosa may be necessary to prevent diabetes. To determine if insulin aerosol-induced γδ T cells display tissue-specific homing, particularly to pancreatic lymph nodes/islets, we labelled unfractionated splenocytes from insulin or control OVA aerosol-treated mice with the fluorescent dye CFSE and adoptively transferred them into syngeneic recipients. By flow cytometry, we found that γδ T cells were widespread and not reproducibly localized to any particular site after aerosol insulin. As it appears that only a small subfraction of CD8 γδ T cells are responsible for the suppression of adoptive transfer of diabetes by splenocytes from insulin aerosol-treated mice (32), CD8 and TCR γδ may not be adequate as markers for monitoring the trafficking of regulatory cells, for which a specific marker is required. Alternatively, CD8 γδ T cells may exert their regulatory function in multiple sites.

A 'non-immune' mechanism could also conceivably contribute to the effect of aerosol insulin to decrease diabetes incidence, for example migration of CD8 γδ T cells to islets to inhibit β-cell apoptosis or promote β-cell regeneration, analogous to the local action of IELs on intestinal epithelial cells (18, 20). A subset of γδ T cells (Vγ9 Vδ2) was implicated in protecting against β-cell destruction by finding that the number of these cells in the circulation remained higher in islet cell antibody-positive individuals who did not progress to diabetes compared to those who did (75).

Through their participation in homeostatic regulation of epithelial integrity in the gut, γδ T cells may also play a role in preventing absorption of protein antigens that might potentially trigger autoimmunity. There is weak epidemiological evidence that early exposure to dietary antigens in cows' milk or short duration of breast feeding is associated with an increased risk of type 1 diabetes, and children with recent-onset diabetes or at risk for type 1 diabetes have exaggerated immune

Fig. 3. Possible regulatory T-cell mechanisms.



responses to cows' milk proteins (reviewed in (76)). A T-cell line isolated from pancreatic islets of a recently diagnosed human showed preferential binding to mucosal vascular endothelium (77), implying a mucosal origin and/or homing preference for islet-infiltrating lymphocytes. In NOD mice, T cells of mucosal origin infiltrate islets earliest during disease pathogenesis and, after adoptive transfer of 'diabetogenic' spleen cells (78), blockade of the vascular addressin mucosal addressin cell adhesion molecule (MAdCAM-1) prevents disease development (79). It is conceivable that genetic susceptibility operates at the level of the mucosal immune system in individuals who develop diabetes. A postulated protective role for breast feeding could be attributed to cytokines and growth factors in breast milk that contribute to the maturation of intestinal mucosa (80) and conceivably IELs. NOD mice maintained under germ-free conditions have a high incidence of diabetes that is reduced by conventional conditions of housing and feeding (39). Under conventional conditions, bacterial colonization of the intestine is accompanied by an increase in IEL number and maturation of mucosal immune function (40, 41). Furthermore, as immunoreactive insulin is readily detectable in breast milk (L. C. Harrison, unpublished), the generation of regulatory cells to insulin could be a normal developmental process in the infant.

To induce regulatory CD8 γδ T cells, insulin has to be delivered as a whole protein by aerosol or intranasally. The B24-C36 peptide from proinsulin, given intranasally, induced CD4 not CD8 regulatory T cells (33), as did intranasal GAD peptides (35). On the other hand, oral insulin induces regulatory CD4 T cells (31). We propose, therefore, that insulin is not degraded in the naso-respiratory tract and is recognized there by CD8 γδ T cells as an intact protein, either directly or presented by an as yet undefined APC/restriction molecule. This would be consistent with reports (reviewed in (10)) that γδ TCRs recognize long peptides and molecular conformations, rather than short linear peptides recognised by αβ TCR. On the other hand, in the gastrointestinal tract, insulin is degraded to peptides which, like intranasal (pro)insulin peptide, induce regulatory CD4 T cells restricted by conventional MHC class II. This scenario probably rules out an intermediate activated CD4 T cell in the generation of regulatory CD8 γδ T cells, supported by our finding that regulatory CD8 T cells can be generated by aerosol insulin in NOD mice lacking CD4 (33).

Conclusions

- γδ T cells are abundant in epithelia of mucosa and the skin, where they participate in immune defence, immune regulation and tissue homeostasis.
- Evidence from several studies indicates that γδ T cells are important mediators of mucosal tolerance and regulate autoimmunity.
- Mucosal administration of insulin or (pro)insulin peptides to NOD mice delays/prevents diabetes by inducing active tolerance that is transferable by regulatory cells. Oral insulin and intranasal (pro)insulin peptides induce regulatory CD4 T cells, whereas aerosol and intranasal insulin induce regulatory CD8 γδ T cells.
- The mechanisms responsible for induction of γδ T cells by aerosol or intranasal insulin are not fully understood, although this effect of insulin is unrelated to its bioactivity and is therefore based on its recognition as an antigen.
- Only a small fraction of CD8 γδ T cells in splenocytes from insulin aerosol-treated mice are responsible for the suppression of adoptive transfer of diabetes by 'diabetogenic' effector T cells.
- The regulatory function of CD8 γδ T cells may involve their localization in pancreatic lymph nodes and production of IL-10. However, given their unique physiological role, γδ T cells could also exert their regulatory function by several other mechanisms.
- To induce regulatory CD8 γδ T cells, insulin has to be delivered by aerosol or intranasally, not orally. This implies that regional differences in mucosal compartments influence the degradation, processing and presentation of insulin and subsequent induction of regulatory T cells. Induction of CD8 γδ T cells requires conformationally intact but not biologically active insulin. We propose that, in contrast to aerosol or intranasal insulin, oral insulin is degraded in the gut to MHC class II-restricted epitopes that induce regulatory CD4 not CD8 γδ T cells.

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